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(54) Title: METHOD OF ENHANCING DIFFERENTIATION AND SURVIVAL OF NEURONAL PRECURSOR CELLS

(57) Abstract

Method of enhancing the differentiation of neuronal precursor cells into NGF-dependent neuronal cells, which method includes treating precursor cells with a combination of a member of the FGF family and a member of the CTNF family to enhance their effectiveness and survival in transplantation therapy for the treatment of diseases such as Parkinson's disease.

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Method of Enhancing Differentiation and Survival of Neuronal Precursor Cells

BACKGROUND OF THE INVENTION

The present invention relates to a method of treating neuronal precursor cells to cause them to differentiate into neuronal-type cells. Such method is useful, e.g. to enhance the survival and functionality of cells used in striatal or other transplantation treatments or for the treatment of tumors.

The development and maintenance of the vertebrate nervous system depends on specific proteins, termed neurotrophic factors, originally defined by their ability to support the survival of neuronal populations (Snider and Johnson, 1989, Ann. Neurol. 26:489). Neurotrophic factors have also been implicated in processes involving the proliferation and differentiation of neurons (Cattaneo and McKay, 1990, Nature 347: 762-765; Lindsay and Harmar, 1989, Nature 337: 362-364), and they may play additional, thus far unexplored, roles both within as well as outside of the nervous system.

The first neurotrophic factor identified was nerve growth factor (NGF). Based on the amino acid sequence of the mouse NGF protein (Angeletti, et al., 1973, Biochemistry 12:100-115) DNA sequences coding for mouse and human NGF have been isolated (Scott et al., 1983, Nature 302:538-540; Ullrich et al., 1983, Nature 303:821-825).

Brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) have recently been molecularly cloned and shown to be additional members of the NGF family by virtue of their sequence homology (Leibrock et al., 1989, Nature 341:149-152; Hohn et al., 1990, Nature 344:339-341; Maisonpierre et al., 1990a, Science 247:1446-1451; Rosenthal et al., 1990, Neuron 4:767-773; Ernfors et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:5454-5458; Jones and Reichardt, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:8060-8064; Hallbook, et al., 1991, Neuron 6:845-858; Ip et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:3060-3064). This family of proteins plays an important role in both the developing and the adult vertebrate nervous system, where these proteins support neuronal survival.

NGF supports the development and maintenance of peripheral sympathetic and neural crest-derived sensory neurons (reviewed in Thoenen and Barde, 1980, Physiol. Rev., 60: 1284-1325; Levi-Montalcini, 1987, Science, 237: 1154-1162). In the brain, NGF has been shown to support basal forebrain cholinergic neurons (reviewed in Whittemore and Seiger, 1987, Brain Res., 434: 439-464; Thoenen et al., 1987, Rev. Physiol. Biochem. Pharmacol., 105: 145-178; Ebendal, 1989, Prog. Growth Factor Res. 1: 143-159).

The effects of the neurotrophins are mediated by their interaction with specific receptors present on sensitive cells. NGF may play a role in early development that is distinct from its function as a neurotrophic factor. In agreement with this possibility, NGF has recently been shown to control proliferation

and differentiation of E14 rat embryonic striatal precursor cells in culture (Cattaneo and McKay, 1990, Nature, 347: 762-765).

The receptor and signal transduction pathways utilized by NGF have been extensively studied, in large part due to the availability of a pheochromocytoma cell line (PC12) which differentiates in response to NGF (Greene and Tischler, 1976, Proc. Natl. Acad. Sci. U.S.A. 73:2424). These studies have resulted in the cloning of a membrane-bound protein (designated "LNGFR" for low-affinity NGF receptor) which binds NGF with relatively low affinity (Chao et al., 1986, Science 232:518-521; Radeke et al., 1987, Nature 325:593-597). In addition to the LNGFR another protein (designated "HNGFR" for high-affinity NGF receptor), which is involved in forming a higher affinity binding site for NGF, is apparently required to initiate NGF-induced signal transduction (Zimmerman et al., 1978, J. Supramol. Struc. 9:351-361; Sutter et al., 1979 in Transmembrane Signalling (N.Y. Alan Liss) pp. 659-667; Bernd and Greene, 1984, J. Bio. Chem. 259:15509-15516; Hempstead et al., 1989, Science 243:373-375). This HNGFR is phosphorylated on tyrosine in response to NGF, and apparently contains intrinsic tyrosine kinase activity (Meakin and Shooter, 1991a, Neuron 6:153-163).

Recent studies have revealed that the product of the trk proto-oncogene, which resembles a growth factor receptor (i.e., it is a transmembrane protein containing an intracytoplasmic tyrosine kinase domain) for which no ligand had been identified, is rapidly phosphorylated in response to NGF treatment in PC12 cells (Kaplan et al., 1991, Nature 350:156-160; Klein et al., 1991, Cell 65:189-197) and can directly bind

NGF with relatively high affinity when expressed in heterologous cells (Klein et al. supra). This finding, together with the restricted neuronal distribution of the trk protein in vivo, suggests that trk (now known as trkA) is the component of the HNGFR responsible for initiating NGF signal transduction.

Definitive studies have indicated that BDNF and NT-4 primarily use the trkB receptor (Squinto, et al. 1991, Cell 65: 885-893); Ip, et al. 1992, Proc. Natl. Acad. Sci. U.SA. 89:3060-30634) and NT-3 is the preferred ligand for trkC (Lamballe, et al. 1991, Cell 66:967-979). In addition, $p75^{LNGFR}$ has been shown to bind with low-affinity to all members of the neurotrophin family (Rodriguez-Tebar et al, 1990, Neuron 4: 487-492; Squinto, et al. 1991, Cell 65: 885-893; Holbook et al., 1991, Neuron 6:845-858).

Ciliary neurotrophic factor (CNTF) was initially identified, purified, and molecularly cloned based on its ability to support the survival of parasympathetic neurons from the chick ciliary ganglion neurons in vitro (Adler et al., 1979, Science 204:1434-1436; Lin et al., 1989, Science 246:1023-1025; Stockli et al., 1989, Nature 342:920-923). CNTF shows no sequence homology, and does not appear to share any receptor components, with the NGF family of neurotrophic factors (Lin et al., 1989, Science 246:1023-1025; Stockli et al., 1989, Nature 342:920-923).

CNTF can enhance the survival of sensory neurons (Skaper and Varon, 1986, Brain Res. 389:39-46), motor neurons (Sendtner, et al. 1990, Nature 345:440-441; Arakawa et al, 1990, J. Neurosci. 10:3507-3515; Oppenheim et al. 1991, Science 251:1616-1618) pre-ganglionic sympathetic spinal cord neurons

(Blottner et al. 1989, Neurosci. Lett. 105:316-320) and hippocampal neurons (Ip et al 1991, J. Neurosci. 11: 3124-3134). In addition to its neuronal capabilities, CNTF can inhibit proliferation and enhance cholinergic properties of neuronal precursors from the sympathetic ganglion (Ernsberger et al 1989, Neuron 2:1275-1284;) effect cholinergic differentiation of mature sympathetic neurons (Saadat, et al., 1989, J. Cell Biol. 108: 1807-1816) and cause the astrocytic differentiation of O-2A glial progenitor cells (Lillien et al.,1988, Neuron 1:485-494). Human CNTF has been molecularly cloned (Masiakowski, et al. 1991, J. Neurochem. 57:1003-1012.

LIF (Leukemia Inhibitor Factor) and Oncostatin M are broadly acting factors that can inhibit the proliferation and induce the differentiation of the murine myeloid leukemia cell line, M1 [Rose and Bruce, Proc. Natl. Acad. Sci. 88: 8641-8645 (1991)]. Recent analysis suggest these hematopoietic cytokines are distant stuctural relatives of CNTF (Bazan, 1991, Neuron 7: 197-208.; Rose and Bruce, 1991, supra.) It has recently been shown that CNTF, LIF and Oncostatin M share signaling pathways that involve the IL-6 signal transducing receptor component gp130, and that LIF induced tyrosine phosphorylations and gene activations in neuronal cels are indistinguishable from responses induced by CNTF (Ip, et al. 1992, Cell 69:1121-1132).

Growth factors known as fibroblast growth factors constitute a family of structurally related factors (the "FGF family") that are unrelated to the NGF family (the "neurotrophins") or to the CNTF family of factors (Burgess and Maciag, 1989, Ann. Rev. Biochem. 58:575; Goldfarb, 1990, Cell

Growth and Differentiation 1:439-445; Thompson, et al. 1991 "Fibroblast Growth Factor" in Methods In Enzymology 198:96-171). They are widely distributed in tissues and have potent mitogenic activity on a variety of cell types including mesoderm and neuroectoderm-derived cells types as well as epithelial cells.

Members of the FGF family that have thus far been identified include FGF-1 [acidic fibroblast growth factor (aFGF); Burgess and Maciag, 1989, Ann. Rev. Biochem. 58:575], FGF-2 [basic fibroblast growth factor (bFGF); Abraham, et al. 1986, EMBO J. 5:1523; Kurokawa, et al. 1987, FEBS Lett. 213:189], FGF-3 (int-2), FGF-4 (HST), the product of the genes known as FGF-5 and FGF-6 (Marics, et al., 1989, Oncogene 4:335), and FGF-7 [keratinocyte growth factor (KGF); Finch, et al. 1989, Science 245:752]. Acidic and basic fibroblast growth factor (aFGF and bFGF, respectively), in addition to their potent mitogenic properties for a variety of cells of mesodermal origin, support the survival of a number of neuronal cells (Walicke et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:3012-3016; Morrison et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:7537-7541; Hatten et al., 1988, Dev. Biol. 125:125:280-289) including chick parasympathetic ganglion neurons.

The sympathoadrenal sublineage of the neural crest include the neurons of the sympathetic ganglia, SIF (small intensely florescent) cells and the chromaffin cells of the adrenal medulla (Landis and Patterson, 1981, Trends Neurosci. 4: 172-175; Doupe et al. 1985, J. Neurosci. 5:2143-2160). Although these cell types are morphologically distinct, they can be interconverted using the appropriate developmental signals

(Doupe, et al. 1985, J. Neurosci. 5:2143-2160). A cell identified in primary cultures of embryonic rat adrenal glands appears to be the progenitor from which chromaffin, SIF and sympathetic neurons are derived. Under conditions, such as in the adrenal gland, where glucocorticoids are present, the progenitor cell can develop into a chromaffin cell, which is an endocrine (generally epinephrin)-secreting cell. Under conditions where glucocorticoids are absent, progenitor cells, as well as chromaffin cells, can develop into sympathetic neurons (Anderson and Axel, 1986, Cell 47:1079-1090; Anderson, 1988, NATO ASI Series, Vol. 22:188-198).

Progenitor cells of the sympathoadrenal lineage coexpress both neuronal and chromaffin cell markers. Monoclonal antibodies against sympathoadrenal antigens have been used to isolate such progenitor cells from embryonic sympathetic ganglia (Carnahan and Patterson, J. Neurosci. 11:3520-3530).

Immortalized cell lines have been established from rat sympathoadrenal progenitor cells. (Birren and Anderson, 1990; Neuron 4:189-201.) These cell lines are known as MAH (v-myc infected, adrenal-derived, HNK-1-positive) cells (HNK -1 is a specific cell surface antibody that, within the developing adrenal gland, exclusively labels tyrosine hydroxylase positive cells of the sympathoadrenal lineage).

NGF in combination with a factor in heart cell-conditioned medium can induce mature chromaffin cells to differentiate into sympathetic neurons (Unsicker et al, 1978; Proc. Natl. Acad. Sci. U.S.A. 75:3498-3502; Doupe et al, 1985, J. Neurosci. 5:2119-2142). NGF also causes neuronal differentiation

in PC12 cells, a cell line of adrenal medullary origin (Greene and Tischler, 1976, Proc. Natl. Acad. Sci. U.S.A. 73:2424-2428).

Acidic FGF and bFGF induce similar responses to NGF in PC12 cells, including neurite outgrowth and the induction of neuron-specific mRNAs (Leonard, et al, 1987, Mol. Cell. Biol. 7:3156-3167; Stein et al., 1988, Dev. Biol. 127:316-325). Like NGF, bFGF will induce cell division and neurite outgrowth from cultured neonatal rat adrenal chromaffin cells; the neurite outgrowth, but not the proliferation is inhibited by dexamethasone (Stemple, et al. 1988, Neuron 1:517-525). Unlike NGF, however, bFGF will not support the survival of chromaffin cell-derived sympathetic neurons, although bFGF has been shown to induce a dependence on NGF to such cells (Stemple, et al. supra).

Studies using MAH cells support the hypothesis that FGF or some other similarly acting factor is the initial determinant of neuronal differentiation in the sympathoadrenal lineage, and that developing neurons acquire a dependence on NGF as they differentiate, as reflected in the induction of NGF receptor gene expression by FGF (Birren and Anderson, 1990;id). Although these studies suggest that FGF induces NGF dependence, this only occurs in a small portion of FGF treated MAH cells. FGF promotes cell growth and survival of MAH cells in the presence of dexamethasone; removal of dexamethasone results in the death of MAH cells, although a very small percentage of the FGF treated cells can survive and differentiate in the absence of dexamethasone but in the presence of NGF.

Parkinson's disease results from the degeneration of a discrete population of neurons located in the substantia nigra of the brain. Neurons in the substantia nigra produce the neurotransmitter dopamine, and form synapses with neurons in the striatum. Loss of dopamine in the striatum results in symptoms characteristic of Parkinson's, such as tremors, rigidity, difficulty in standing and slowness of movement.

Treatment of Parkinson's has thus far been based on either treatment with the dopamine precursor L-dopa, or replacement of dopamine producing cells. Such tissue is generally derived from fetal CNS tissue or from the adrenal gland. However, transplantation into the substantia nigra requires the growth of axons through the brain to connect with their targets in the striatum; such growth is unlikely in the adult brain. Thus, dopamine-producing cells are generally grafted closer to their targets in the striatum.

Chromaffin cells of the adrenal gland secrete catecholamines. Maintenance of their endocrine phenotype in the adrenal gland is under the control of the glucocorticoids. Chromaffin cells cultured in the absence of glucocorticoids are known to be able to differentiate into a neuronal-type phenotype, thus suggesting their use in transplants to innervate brain tissue (Olson, L. 1970. Histochemie 22:1-7; Olson, et al. 1980, Exp. Neurol. 70: 414-426). CNTF also promotes process outgrowth from chromaffin cells (Shults, 1992, "Parkinson's Disease" Neurologic Clinics 10:567-593). Administration of NGF, in conjunction with adrenal medullary implant, is known to lead to better cell survival and more functional effect (Strombert, et al.

1985. Exp. Brain Res. 60:335-349; Strombert, et al. 1985, Cell Tissue Res. 241:241-249). Despite such reports, adrenal medullary implants have resulted in very limited success because effective treatment requires both the survival of the cells upon implantation, and the subsequent conversion of the grafted adrenal medulla cells into dopaminergic neurons.

SUMMARY OF THE INVENTION

The object of the present invention is to provide a method of producing sympathetic neurons from neuronal precursor cells.

A further object of the present invention is to provide a method of producing adrenal gland derived dopaminergic neurons.

Another object of the present invention is to enhance the differentiation and survival of cells used for transplantation treatment of Parkinson's disease, as well as other diseases or disorders that can be treated using transplantation therapy.

Another object of the invention is to provide a method of causing the differentiation of tumor cells, thereby inhibiting or retarding their growth.

These and other object are achieved by the present invention, which provides a method of effecting the growth, differentiation and survival of neuronal precursor cell lines by treating such cells with a combination of a member of the FGF growth factor family in combination with one or more members

of the NGF family or the CNTF family of factors. Preferably such effects are used in the context of therapies for the treatment of diseases which involve loss or a defect in neuronal tissue, such as Parkinson's disease or Alzheimers.

In one embodiment of the invention, cells derived from the adrenal gland are treated with a combination of basic fibroblast growth factor and ciliary neurotrophic factor to cause such cells to differentiate and, concomitantly, become dependent on nerve growth factor. Such cells are therapeutically useful for the treatment of, for example, Parkinson's disease.

In another embodiment, the method of the present invention is used to treat tumor cells by promoting their differentiation and inhibiting their growth.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Cell growth of MAH cells treated with CNTF, FGF or both factors. Open circles indicate control (untreated) cells; closed squares indicate the effect with treatment of CNTF alone; open squares indicate treatment with FGF alone; closed triangles indicate treatment with CNTF and FGF in combination.

A. Cells were treated with CNTF alone, FGF alone or a combination of both factors for 1-4 days prior to 3H-thymidine uptake assay.

B. Total number of cells were counted at the end of a week treatment with CNTF (C), FGF (F), or both factors (C+F).

C. Cells pretreated with FGF (F) alone or CNTF+FGF (C+F) for 1 week were washed free of the factors and dexamethasone,

and NGF was added to continue culture for 1 week or 2 weeks. Total cell number following these treatments were determined.

Figure 2. Collaboration of CNTF, FGF and NGF in the making of a neuron.

A. MAH cells were incubated in the presence of dexamethasone (Control), plus CNTF alone, FGF alone, or a combination of both factors (CNTF+FGF) for a period of 4 days (top row). Following such a pretreatment period of 7 days, cells were washed free of the factors and dexamethasone, and NGF was added for the rest of the culture period (bottom row).

B. Four representative fields were depicted for FGF-pretreated cells (top row), or CNTF+FGF pretreated cells (bottom row). Both were cultured in the presence of NGF following withdrawal of dexamethasone.

C. Cells were pretreated with CNTF+FGF for 7 days, and washed free of the factors and dexamethasone. Each of the 4 neurotrophins NGF, BDNF, NT-3 and NT-4 was added to test for their ability to collaborate with FGF and CNTF to make neurons.

Figure 3. CNTF, FGF and NGF collaborate to make postmitotic neurons that are neurofilament-immunopositive.

A. MAH cells were pretreated with CNTF and FGF for 1 week, and were then washed free of the factors and dexamethasone. The cells were then cultured in the presence of NGF for 1 week to 5 weeks. The pictures were taken from the same field at the various times indicated to demonstrate these cells are postmitotic.

B. Neurofilament staining was performed on MAH cells at the end of a 7 day pretreatment with CNTF, FGF or both factors, or followed the addition of NGF.

Figure 4. Changes in gene expression in CNTF and FGF pretreated MAH cells.

MAH cells were treated with CNTF, FGF or both factors for 7 days, and gene expression for LNGFR, trkA and c-myc were examined.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based, in part, on the discovery by applicants that the treatment of neuronal precursor cells with a combination of members from the structurally and functionally unrelated neurotrophic factor and growth factor families results in the differentiation of the cells to a neuronal, NGF-dependent phenotype.

As described above, it was previously known that treatment of adrenal-gland derived chromaffin cells with bFGF resulted in the differentiation of a small percentage of such cells into neuronal-type cells, and that such differentiated cells became dependent for survival on NGF. As described herein, applicants have discovered that treatment of adrenal-gland derived precursor cells with the combination of one or more members of the NGF family or the CNTF family in conjunction with one or more members of the FGF family results in the differentiation of

a significantly higher portion of the cells into neuronal-type cells.

Thus, according to the invention, neuronal precursor cells are treated with a combination of one or members of the FGF family selected from the group consisting of FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6 and FGF-7 in conjunction with one or more factor from the CTNF family, which includes CNTF, Oncostatin M and LIF, or from the NGF-family, which includes NGF, BDNF, NT-3, and NT-4 . In each instance, the member of the FGF, CNTF or NGF family used may consist of the mature protein, or an analog or biologically active derivative thereof. Members of a family are those factors that share either a common receptor component, such as CNTF and LIF, or members that share a common receptor.

Neuronal precursors include any cell capable of differentiating into a neuronal type cell. As used herein, neuronal precursors include both progenitor cells, as well as cells such as, for example, chromaffin cells and SIF cells, which, although not considered progenitor cells for sympathetic neurons, can be converted into neuronal type cells given the appropriate signals. Neuronal precursors include immortalized human or animal cell lines or genetically engineered cells capable of differentiating and expressing a neuronal phenotype in response to a neurotrophic or growth factor.

Neuronal precursors may be isolated, for example, from embryonic ganglia using specific markers such as tyrosine hydroxylase or by isolating cells that display both neuron- and chromaffin-cell specific markers (Carnahan and Patterson, 1991,

J. Neuroscience 11:3493-3506; Carnahan and Patterson, 1991, J. Neuroscience 11: 3520-3530).

In a preferred embodiment, ciliary neurotrophic factor is used, in combination with FGF, to promote the differentiation of adrenal gland derived precursor cells, such as MAH cells. Co-treatment with these factors results in conversion of a large number of cells to a neuronal phenotype, as measured by expression of neurofilament. As distinguished from treatment with either factor alone, cotreatment of the cells results in the marked induction of trkA and $p75^{LNGFR}$ and concomitant reduction in c-myc expression, indicating enhanced receptivity of the cells to NGF.

Cells treated according to the invention may be used, for example, in place of fetal cells or adrenal chromaffin cells, in transplantation therapy for Parkinson's disease. Transplanted chromaffin cells are known to be able to differentiate into a neuronal-type phenotype and to innervate brain tissue (Olson, L. 1970. Histochemie 22:1-7; Olson, et al. 1980, Exp. Neurol. 70: 414-426). Further, administration of NGF, in conjunction with adrenal medullary implant, is known to lead to better cell survival and more functional effect (Strombert, et al. 1985. Exp. Brain Res. 60:335-349; Strombert, et al. 1985, Cell Tissue Res. 241:241-249). Cells pretreated according to the present invention would express NGF binding receptors upon transplantation, and, therefore would be expected to have a better chance of surviving within the striatal environment. Accordingly, the present invention contemplates pretreatment of CNTF/bFGF treated cells with NGF prior to transplant, or co-injection of NGF

or NGF-producing cells in combination with CNTF/bFGF pretreated cells.

Data described herein demonstrates how multiple factors derived from different families of proteins can interact in complex ways to effect the growth and differentiation of neuronal precursors. Strikingly, these interactions do not result in simply the enhancement of the effect of one of the factors, but rather a differentiation process that is qualitatively much different from that resulting from treatment with any one factor alone.

Example 1. Effect of CNTF and bFGF on Cell Growth and Morphology

Materials and Methods

Cell culture

MAH cells were maintained in culture as previously described (Birren et. al., 1990, Neuron 4: 189-201). Briefly, cells were plated onto dishes precoated with poly-D-lysine (100 microgram/ml) and laminin (10 microgram/ml), at a low density of 5,000/35mm well. The medium used was modified L15-CO2 medium supplemented with 10% fetal bovine serum (FBS) and dexamethasone (5micromolar). Cells were treated with FGF (10ng/ml) alone, CNTF (10ng/ml) alone, or a combination of the two factors (10ng/ml) for 7 days in the presence of dexamethasone. the cells were then washed free of factors and dexamethasone, and maintained in the presence of NGF for 1-5

weeks. In control cultures withdrawn from dexamethasone, the cells died within 4-5 days.

3H-thymidine incorporation assay and cell counts

For 3H-thymidine incorporation assay, MAH cells were treated with CNTF, FGF, or both factors for 1-4 days, and 3H-thymidine (NEN-NET-027E purchased from New England Nuclear, MA) was added at a final concentration of 1microcurie/ml and incubated for 4 hours at 37°C. Cells were then washed three times with phosphate-buffered saline (PBS), lysed with NaOH (0.5N) for 2hours at room temperature and 3H-DNA was counted. Total number of cells was also counted by hemocytometer at the end of a 1 week treatment with CNTF, FGF, or both factors, and followed by a 1 week or 2 weeks subsequent treatment with NGF.

Results

As compared to untreated MAH cells, FGF resulted in an increase in cell number, CNTF resulted in a decrease in cell number, whereas a combination of the two factors resulted in intermediate effects (Figures 1A and 1B). In addition to the intermediate effects seen with the combined treatment, cell morphology was notably different in the cells treated with both factors as compared to cells treated with either factor alone (Figure 2A, top row). Cells treated with FGF alone formed large flat colonies with neurites, cells treated with CNTF alone formed small and tightly clustered colonies without neurites, whereas the FGF and CNTF in combination resulted in dispersed colonies consisting of phase-bright neurite bearing cells. Removal of

dexamethasone from these cultures resulted in the complete death of all the MAH cells regardless of prior treatment. However, addition of NGF following the removal of dexamethasone resulted in dramatically different responses depending on the prior treatment. No cells survived if the prior treatment consisted solely of CNTF, small numbers survived if the prior treatment consisted solely of FGF, whereas large numbers of cells resembling neurons resulted from NGF addition to cultures previously treated with both FGF and CNTF (Figure 1C, second row of Figure 2A and Figure 2B). The NGF effect was quite specific in that none of the other neurotrophins (BDNF, NT-3 and NT-4) could rescue any of the FGF/CNTF treated MAH cells (Figure 2B). Time course studies revealed that the largest number of NGF-dependent cells could be generated following six days of FGF/CNTF treatment, and that the continued presence of FGF and CNTF did not effect the extent of NGF-dependent survival following dexamethasone withdrawal (not shown). The percentage of cells surviving in NGF following combined FGF/CNTF treatment was about 2-3% of those present at the time of dexamethasone withdrawal, and this number did not change during prolonged NGF treatment (Figure 1C and 3A). However, the neuritic arborization became more elaborate with time in NGF, and the cells became larger and more phase bright and all expressed neurofilament protein (Figures 3A and 3B). The observations indicated that the cells surviving in the presence of NGF displayed characteristics similar to those of post-mitotic neurons cultured in the presence of NGF.

Example 2. Expression of Neuronal Markers

Materials and Methods

Neurofilament staining

For neurofilament staining, cells were rinsed, and blocked with normal serum prior to incubation with primary antibody specific for neurofilament (RT97, 1:1000 dilution) at 4°C overnight. Cells were then incubated with the biotinylated secondary antibody, followed by avidin:Biotin:Peroxidase complex, and nickel-sulfate intensification of DAB reaction.

RNA isolation and analysis

MAH cells were plated at a density of 100,000-300,000 on 100mm dishes, and treated with CNTF, FGF, or both factors for 7 days. Total RNA was prepared by the guanidinium thiocyanate method (Chomczynski, 1987, Anal. Biochem. 162: 156-159). Ten micrograms of RNA from each sample were electrophoresed on a formaldehyde agarose gel, transferred to a nylon membrane (MSI), and hybridized to 32P-probes labelled by random oligo-priming (Stratagene). The probes used included p75LNGFR, trkA and c-myc.

Results

To further define the early changes distinguishing combined FGF/CNTF treatment from treatment with either factor alone, we examined the MAH cells for their expression of neuron-specific markers following the various treatments. Staining with

an antibody specific for neurofilament revealed that untreated or CNTF treated MAH cells did not express neurofilament. In contrast, a very small percentage of FGF treated MAH cells expressed neurofilament protein, whereas a substantial portion of MAH cells treated with both FGF and CNTF displayed neurofilament expression. Similarly, examination of a number of relevant gene markers revealed that co-treatment with both CNTF and FGF resulted in marked induction of trkA and $p75^{LNGFR}$ mRNA, and a concomitant reduction in c-myc expression (Figure 4). Analysis of these protein and RNA markers defines changes in gene expression which distinguish FGF/CNTF co-treatment from treatments involving either factor alone. Thus combined treatment with FGF/CNTF resulted in inhibition of proliferation as well as enhanced neuronal phenotypic expression in an adrenal gland derived precursor cell.

CLAIMS

1. A method of causing the differentiation of neuronal precursor cells comprising treating the cells with a member of the FGF family and at least one member of the CNTF family or NGF family of factors.

2. The method according to claim 1 wherein said member of the FGF family is one or more factor selected from the group consisting of FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6 and FGF-7.

3. The method according to claim 1 wherein said member of the CNTF family is selected from the group consisting of ciliary neurotrophic factor, oncostatin M and LIF.

4. The method according to claim 1 wherein said member of the NGF family is selected from the group consisting of BDNF, NGF, NT-3 and NT-4.

5. The method according to claim 1 wherein said neuronal precursor cells are fetal cells.

6. The method according to claim 1 wherein said neuronal precursor cells are selected from the embryonic sympathetic ganglia.

7. The method according to claim 1 wherein said neuronal precursor cells are immortalized progenitor cells.

8. A method of treating a tumor comprising administering a therapeutically effective amount of a member of the FGF family and at least one member of the CNTF family or NGF family of factors.

9. The method according to claim 8 wherein said member of the FGF family is one or more factor selected from the group consisting of FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6 and FGF-7.

10. The method according to claim 8 wherein said member of the CNTF family is selected from the group consisting of ciliary neurotrophic factor, oncostatin M and LIF.

11. The method according to claim 8 wherein said member of the NGF family is selected from the group consisting of BDNF, NGF, NT-3 and NT-4.

12. The method according to claim 11 wherein said neurotrophin is selected from the group consisting of BDNF, NGF, NT-3 and NT-4.

13. A method of treating a neurological disease in a patient in need of such treatment comprising administering, to the patient, an effective amount of neuronal precursor cells which have been

pretreated with a combination of a member of the FGF family in combination with one or more members of the NGF family or the CNTF family of factors.

14. The method according to claim 13 wherein said member of the FGF family is bFGF and said member of the CNTF family of factors is CNTF.

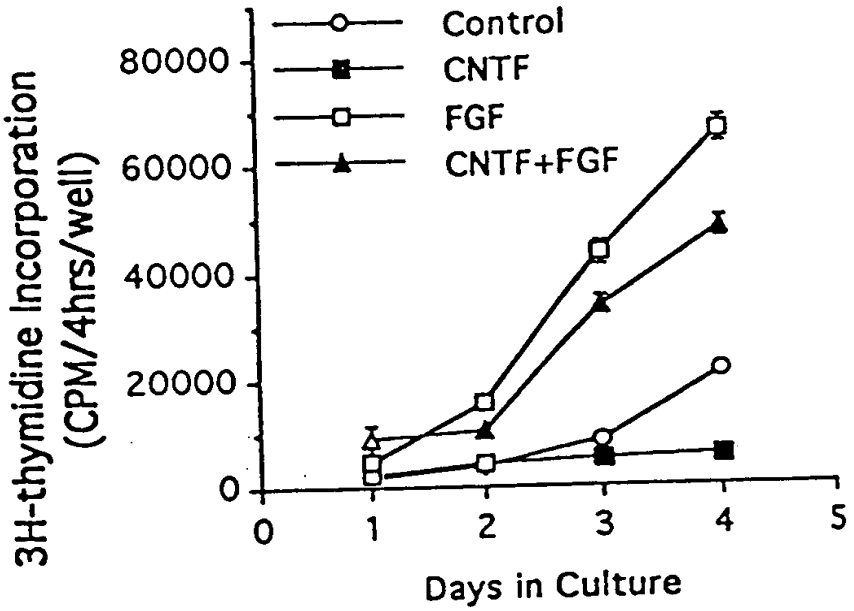
15. The method according to claim 14 wherein said pretreatment is for from one to six days.

16. The method according to claim 13 wherein said pretreated cells are administered in combination with infusions of nerve growth factor.

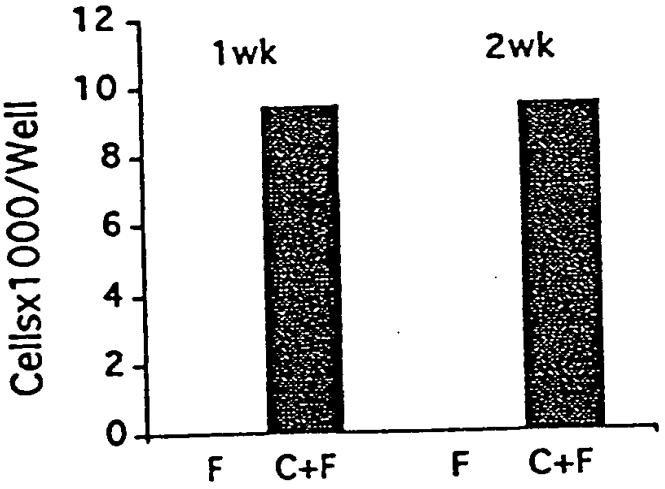
17. The method according to claim 13 wherein said pretreated cells are administered by co-transplantation with nerve-growth factor producing cells.

18. The method according to claim 13 wherein said pretreatment further comprises treatment with NGF.

A.

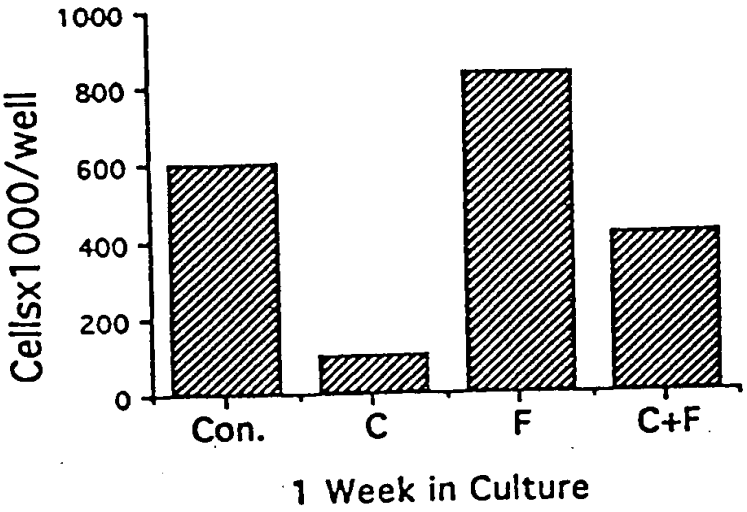


C.

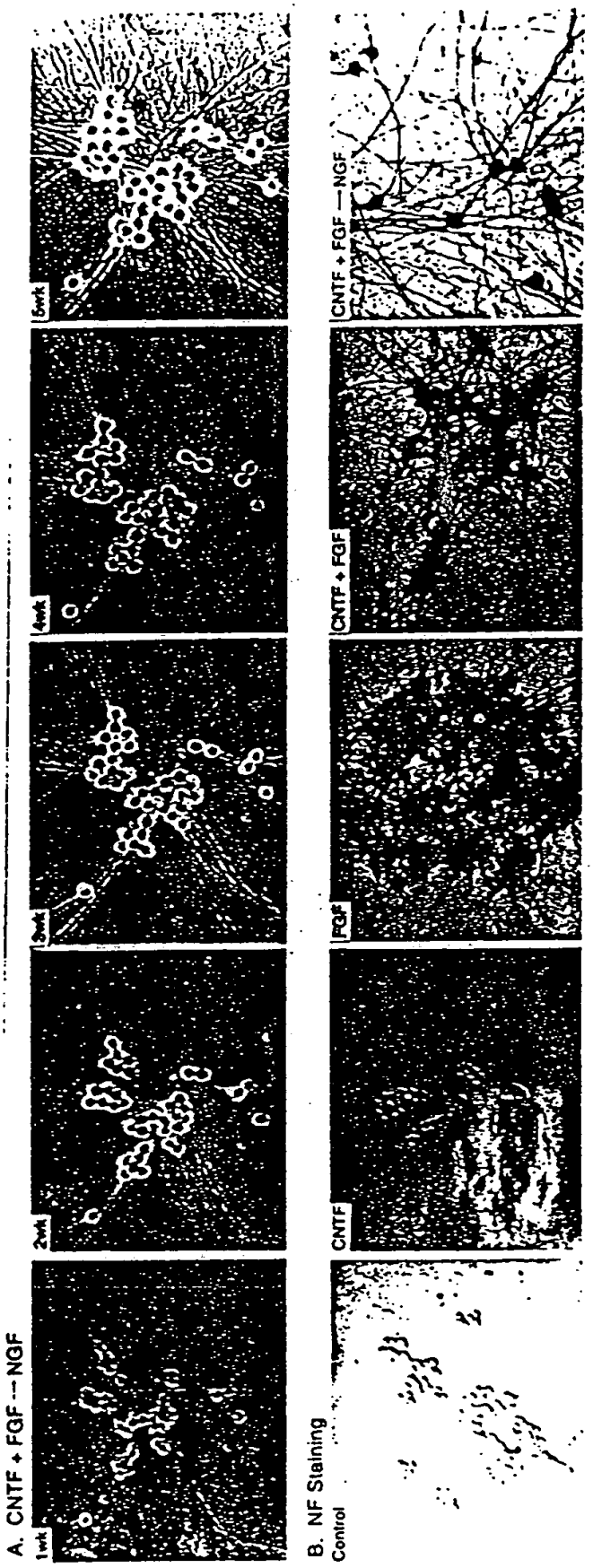


REG 100
FIG. 1
1/7

B.

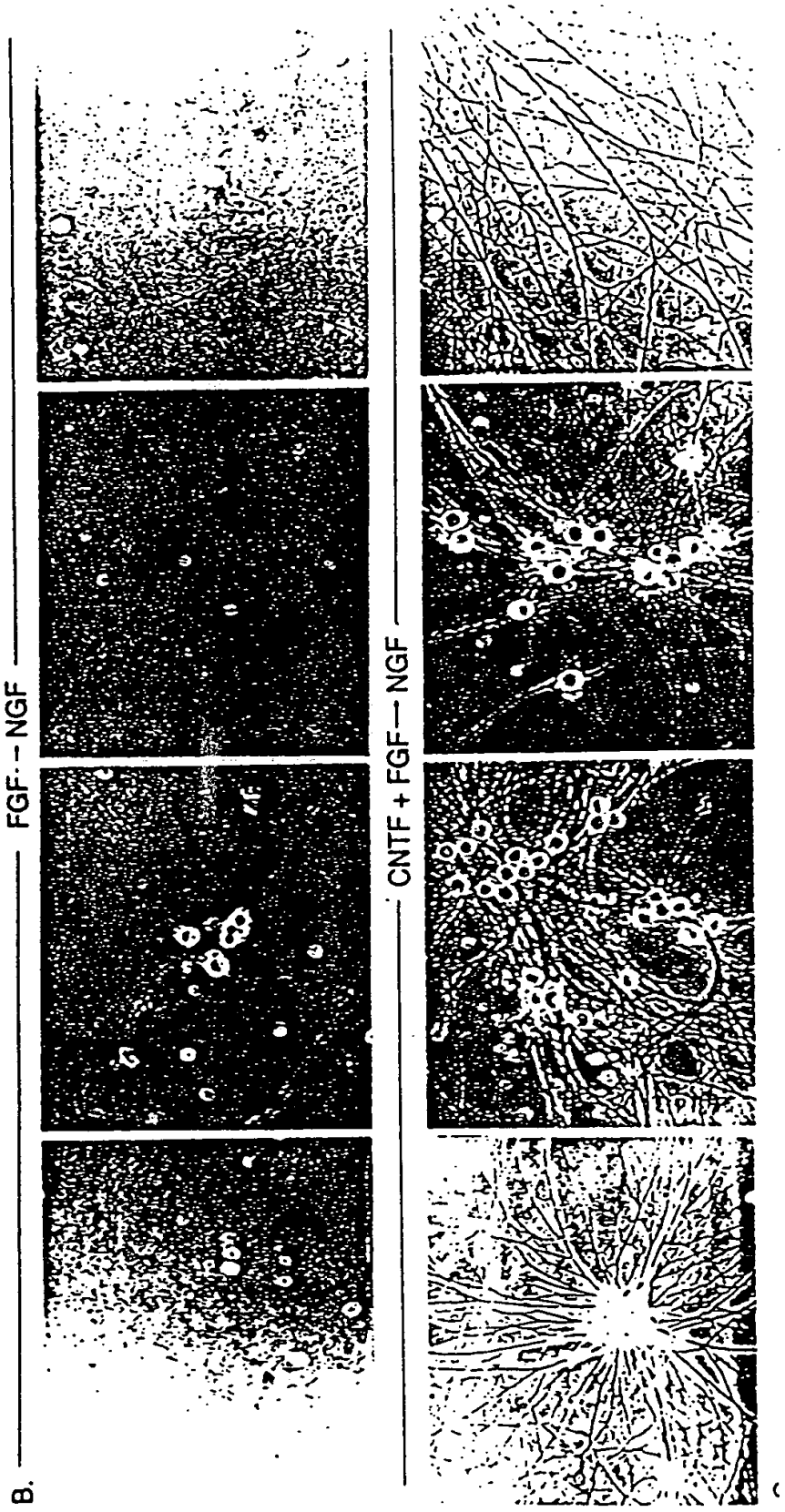


REG 100
FIG. 1
2/7



REG 100
FIG. 2
3/7

SUBSTITUTE SHEET



REG 100
FIG. 3
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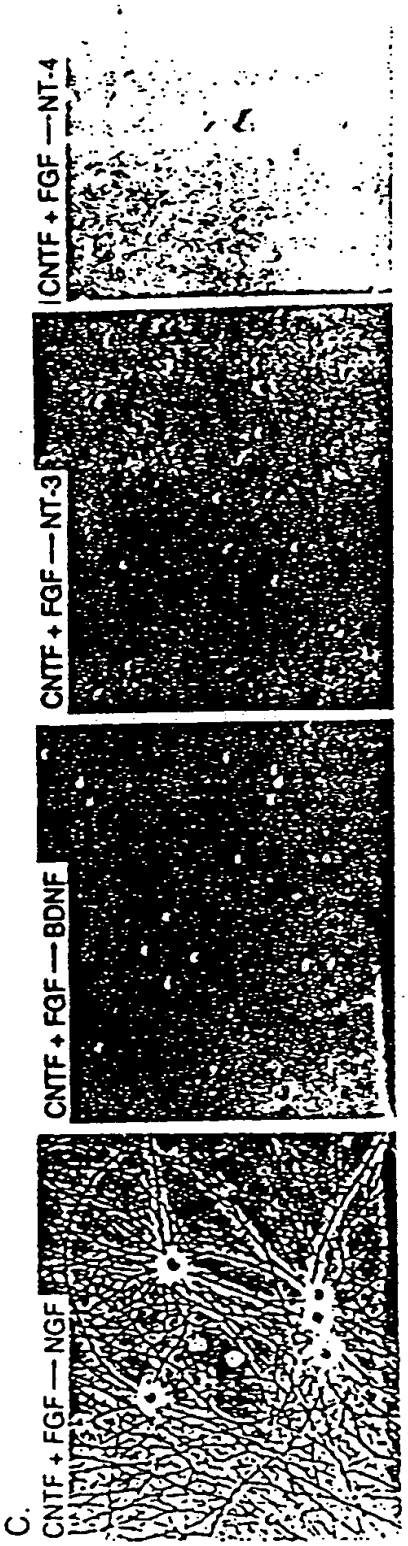
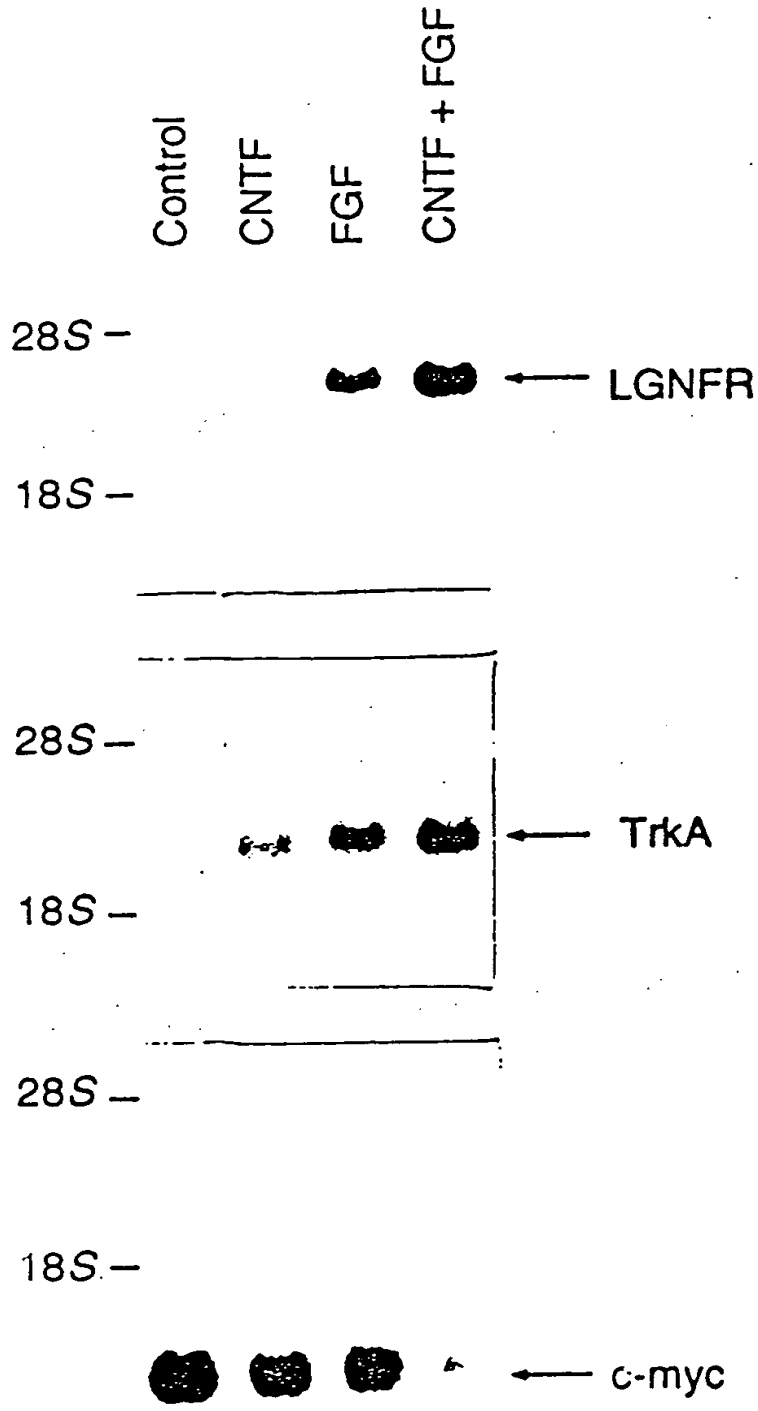


C.

REG 100
FIG. 3



REG 100
FIG. 4
7/7

INTERNATIONAL SEARCH REPORT

Inter mal Application No
T/US 93/07167

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K37/02 C12N5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 04316 (MAX PLANCK INSTITUT FÜR PSYCHIATRIE ET AL.) 4 April 1991 see page 41, line 18 - page 44, line 34 see page 118, line 20 - page 121, line 23	1-3
Y	WO,A,91 03568 (MAX PLANCK GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V. ET AL.) 21 March 1991 see page 55, line 1 - page 58, line 34	1,4
Y	WO,A,90 05138 (THE CHILDREN'S MEDICAL CENTER CORPORATION) 17 May 1990 see page 8, line 4 - line 24	1,2,4

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 November 1993	17 -12- 1993

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	REMPP, G

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DEVELOPMENT vol. 114, no. 3 , March 1992 pages 743 - 754 FRANCOIS GUILLEMOT ET AL. 'RETINAL FATE AND GANDLION CELL DIFFERENTIATION ARE POTENTIATED BY ACIDIC FGF IN AN IN VITRO ASSAY OF EARLY RETINAL DEVELOPMENT' see the whole document	1,2
Y	JOURNAL OF NEUROSCIENCE RESEARCH vol. 25, no. 4 , April 1990 pages 463 - 475 M. MURPHY ET AL. 'FIBROBLAST GROWTH FACTOR STIMULATES THE PROLIFERATION AND DIFFERENTIATION OF NEURAL PRECURSOR CELLS IN VITRO.' see the whole document	1,2
Y	DEVELOPMENTAL BIOLOGY vol. 152, no. 2 , August 1992 pages 363 - 372 JURGEN ENGELE ET AL. 'EFFECTS OF ACIDIC AND BASIC FIBROBLAST GROWTH FACTORS (AFGF, BFGF) ON GLIAL PRECURSOR CELL PROLIFERATION :...' see the whole document	1,2
Y	NEURON vol. 6, no. 6 , June 1991 pages 949 - 955 MAYA SIEBER-BLUM 'ROLE OF THE NEUROTROPHIC FACTORS BDNF AND NGF IN THE COMMITMENT OF PLURIPOTENT NEURAL CREST CELLS' see the whole document	1,4
Y	INTERNATIONAL JOURNAL OF DEVELOPMENTAL NEUROSCIENCE vol. 7, no. 5 , 1989 pages 465 - 473 K. SEIDL ET AL. 'SURVIVAL AND NEURITIC GROWTH OF SYMPATHOADRENAL (CHROMAFFIN) PRECURSOR CELLS IN VITRO' see the whole document	1,4
A	NEURON vol. 1, no. 6 , August 1988 pages 485 - 494 LAURA E. LILLIEN ET AL. 'TYPE-2 ASTROCYTE DEVELOPMENT IN RAT BRAIN CULTURES IS INITIATED BY A CNTF-LIKE PROTEIN PRODUCED BY TYPE-1 ASTROCYTES' see the whole document	1,3

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 93/ 07167

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 8-18 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found muluple invenuons in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invenuon first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.